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Introduction

In 1977 we began a study whose objectives were:

(1) to develop protocol to study the impact of ozone on qualitative characteristics of three crops viz. potato, alfalfa and soybean;

(2) to determine whether ozone had the potential to change the quality of defined characteristics;

(3) to determine the relationship between ozone injury to the receptor viz. the leaf, and the edible organ for each species.

Ozone was selected for this study because it is the most ubiquitous phytotoxic air pollutant in the United States. It is formed photochemically through a series of reactions with by-products of auto exhausts and power plant emissions (17,21). Transport of ozone from urban to rural areas is well documented (58). In 1979 the National Air Quality standard for oxidants changed such that the maximum hourly average was not to exceed 0.12 ppm (rather than 0.08 ppm) more than once a year. The rationale for the altered standard was that this level of oxidant stress would not represent a significant threat to human health or associated environment, including plant life. This decision created an impetus to further identify potential response of plants to ozone.

The crops were selected because they had acknowledged ozone susceptibility (19,20,23,35,37,52). There is evidence that ozone has induced injury to alfalfa, potato, and soybean in the field. For each, the receptor of the gas would be the leaf. The edible portion for alfalfa is the leaf, for soybean - the bean, and for the

potato - the tuber. The choice of these three crops permitted evaluation of direct and indirect effects of ozone on crop quality.

In all studies, plants were exposed to one or repeated acute dosages of ozone. This exposure regimen was selected since it afforded us a more rapid method of determining potential effects, than would have been permitted by a space limiting chronic exposure design. Plants were treated with ozone in a controlled environment chamber in order to insure repeatable results with a minimum of complexing factors.

For each crop, qualitative characteristics were evaluated as their status related to foliar symptom of the plant. Two classes of quality were selected: (i) Factors which contributed to positive value of crop e.g. protein in alfalfa, starch in potatoes. (ii) Factors which reduced quality e.g. glycoalkaloids in potatoes and isoflavonoids in alfalfa.

In the report which follows, we will detail the characteristics selected for each crop, the experimental design and methodologies developed and results achieved.

I. Potato

Many characteristics of the tuber contribute to the quality of this crop. Altered status of various characteristics determine successful use which is made of the potato viz. table stock - boiling vs. baking, or for chipping. Initially we considered characteristics which included yield, total solids, sugars - reducing and sucrose, and glycoalkaloids. Total solids were considered because of their importance to fluffiness of the potato.

Subsequent studies were conducted to determine which of the major components of total solids viz. starch and/or protein, were responsible for any observed changes. Elevated concentrations of reducing sugars are associated with darkening of potato chips. When levels of reducing sugars exceed 2% on a dry weight basis, chips become unacceptably dark (24,28). This would represent a serious commercial threat. Glycoalkaloids are steroidal alkaloids; these compounds are produced in varied amounts in potato tubers and foliage. The levels found in tubers are determined both by genetic potential of the cultivar and by stress factors, both biotic and abiotic (10,39,45,57). Elevated glycoalkaloid levels in tubers have been associated with bitter taste in potatoes and a variety of maladies including diarrhea, teratogenic abnormalities and inhibited cholinesterase function (2,45,55,56).

Experimental Plan

Potato Culture

During May through October, 1977 (Experiment I) and March through August, 1978 (Experiment II) experiments were conducted to determine impact of ozone on yield, total solids, sugar status and tuber glycoalkaloid (TGA) content of

'Norland' and 'Kennebec' cultivars of Irish potato. Tubers of 'Kennebec' (obtained from E. D. Jones, Cornell University, Ithaca, N. Y. 14853) and 'Norland' (obtained from Tobiason Potato Co., Inc., Box 1672, Grand Forks, N. D. 58201) were removed from cold storage (3.5°C), cut into 25-40 g pieces containing 3 or 4 "eyes" and allowed to suberize 10 days to 2 weeks prior to planting. One seed piece per 7.5 liter plastic pot was planted in a steam sterilized 1:1:1 mix of Hagerstown loam:perlite:peat moss in experiment I and mushroom compost casing soil mix in experiment II. Twenty pots of each cultivar were randomly assigned space on a greenhouse bench. Each pot was filled to 75% capacity with the soil mix to allow room for tuber expansion and for addition of soil to "hill" the vines later in the growing season. Every 2 weeks, starting from the second week, 4.5g/l/pot of a soluble 20:20:20 NPK fertilizer (Robert B. Peters Co., Inc., 2833 Pennsylvania St., Allentown, PA. 18101) was applied. The plants were watered to container capacity as needed. Each plant was limited to three vines, which were staked, trained, and pruned to a height of approximately 1.2 m. Insects were controlled by applying 0.3g/plant of Temik 10G (Aldicarb, Union Carbide Corp., 270 Park Ave., New York, N.Y. 10017) 2 weeks after planting and again 60 days later. Foliar fungal diseases were controlled by one foliar application of Manzate 200, 7.5g/3.79 l water (E. I. DuPont de Nemours, Wilmington, Del. 19801), at about 80 days into the growing season. Vines were killed mechanically 10 days prior to harvest of each cultivar. 'Norland' and 'Kennebec' were harvested 120 and 140 days after planting, respectively. In experiment I, the growing season was from May to October. A natural photoperiod was employed, supplemented with fluorescent light providing a maximum light intensity of 25 Klux. The temperature, as measured by a recording thermograph placed

in the canopy of the foliage, ranged from 15° C to 39°C and averaged 24° C. In experiment II the growing season was from March to August; natural light was supplemented with fluorescent light and the photoperiod adjusted weekly to correspond as closely as possible to that of experiment I. The temperature ranged from 15°C to 37°C and averaged 22°C.

Ozone Exposure.

Ten plants of each cultivar, designated as treatment plants, were placed randomly in the exposure chamber the night before fumigation with ozone and were returned to the greenhouse about 6 hr after ozonization. The remaining 10 plants per cultivar were left in the greenhouse and served as non-treated plants. Plants were exposed to ozone in a modified Environmental Growth Chamber as previously described (46). The chamber was maintained at 21°C, 75% relative humidity and a light intensity of 32 Klux. Plants were exposed to 387 µg/m (0.20 ppm) ozone for 3 hr every 2 weeks throughout the growing season starting when the vines were 3 weeks old and ending just prior to harvest. Ozone was produced, monitored and calibrated by previously described techniques (46).

Tuber Sampling

At harvest, the number and weight of individual tubers per plant were recorded; tubers weighing less than 40 g were not included. Tubers were pooled by plant such that each plant represented a sample. For each sample, tubers were diced into 1 cm cubes and mixed. Two - 10 g sub-samples were removed for total solids determinations, 1 - 200 g sub-sample was removed for sugar analysis, and 2 - 20 g sub-samples were removed for TGA analysis. In experiment II an additional 100 g sample was removed for subsequent starch and protein determinations.

Methods of Analysis

Total solids were determined by forced air oven drying at 100°C to a constant weight (26) in 1977 and by lyophilization in 1978.

Sugar extraction and analysis were performed with a modification of the method described by Shaw (43). Approximately 100 ml of liquid were expressed from the tuber sample with a commercial juicerator (40); two milliliters of 1 N sodium bisulfite solution were added. Two 25 ml aliquots were removed and boiled for 5 min. Precipitate was removed from the extracts by centrifugation (15,951 g for 20 min.). Ten ml of supernatant were passed through a Dowex 2-X8 anion exchange column which was washed with 5 ml deionized water; the extract and wash water were then passed through a Dowex 50 W-XB (Na^+ form) cation exchange column which was then washed with 5 ml deionized water. The dimensions of the anion and cation columns were 14 cm X 1.1 cm (length x width). From the 20 ml of extract, 0.2 ml were removed, combined with 0.5 mg perseitol in water as the internal standard (40) and lyophilized.

Derivatives were prepared by adding 0.5 ml of Tri-sil 'Z' (Pierce Chemical Co., Rockford, IL 61105) to the lyophilized sample, shaking occasionally and heating for 30 min. in a sand bath at 60-70°C (51). A 3 µl sample was injected into a Varian Aerograph Gas Chromatograph, Model 1200, equipped with a Chromatographic data system to integrate peaks. A 3% OV-17 on chromosorb W (HP) 80/100 mesh, 1.83 m x 3.2 mm O.D. x 2.1 mm I.D. stainless steel column was used. The injection port and detector temperatures were 230°C and 280°C, respectively. The column temperature was programmed from 110°C at 6°C/min.

Tuber samples for TGA analysis were frozen in liquid nitrogen and stored at -10°C until they were analyzed.

Analysis of TGA.--In experiment I, TGA were analyzed by the method of Fitzpatrick and Osman (15) as modified by Fitzpatrick et al. (13). In experiment II tubers were analyzed for TGA by a method developed for leaf glycoalkaloids (see attached manuscript: Speroni, J. & E. J. Pell) which will be discussed later. Results of TGA analyses were reported on a wet and dry weight basis.

Total protein is being measured in potato tubers by the Coomassie Brilliant Blue procedure (48).

Starch is extracted from lyophilized tuber tissue. First the starch is solubilized in lyophilized tissue with a dimethylsulfoxide solution; then it is hydrolyzed to its glucose components (49). Glucose is quantitated with commercial glucose oxidase-Statzyme Glucose 50 (Worthington Diagnostics, Freehold, N.J. 07728).

Yield, total solids and sugars were statistically analyzed by a weighted least square analysis of variance and treatment means were separated by a multiple comparison test. Tuber glycoalkaloids were analyzed by a 2x2x2 factorial analysis of variance in a completely randomized design.

Results

Ozone exposure of potato plants resulted in foliar tissue collapse and reddish brown lesions of adaxial leaf surfaces. Foliage in the upper third of the plant, with the exception of emerging leaves, was observed to be most sensitive to ozone. 'Norland' foliage exhibited much more severe response than did leaves of 'Kennebec'. Lower leaves of both cultivars responded to ozone by senescing and abscissing earlier than those of control plants.

Ozone significantly reduced the number and weight of tubers per plant for 'Norland' and 'Kennebec' (figure 1). In 1977 and 1978, respectively, 'Norland' sustained a 19% and 21% reduction in mean tuber number and 30% and 20% reduction in mean tuber weight. Tuber numbers of 'Kennebec' were reduced 40% and 32% and mean tuber weights were reduced 54% and 30% in 1977 and 1978, respectively.

Total solids were reduced significantly by ozone in both cultivars (Figure 1). Ozone induced a reduction in total solids in 'Norland' potato tubers of 18% in 1977 and 8% in 1978. Tubers of 'Kennebec' plants exposed to ozone sustained a 27% and 13% reduction in total solids for 1977 and 1978, respectively.

Sucrose and three major reducing sugars viz. fructose, α- and β-glucose, were detected in tuber tissue. Tubers from 'Kennebec' and 'Norland' plants exposed to ozone had a significantly greater concentration of total reducing sugars when these compounds were calculated on a dry weight basis. When the data were pooled by year and only impact of ozone on sugars was considered, we observed that the concentrations of fructose, α- and β-glucose were significantly greater in tubers of 'Kennebec' harvested from exposed plants compared to control plants. The latter two reducing sugars were significantly higher in tubers harvested from ozonized 'Norland' compared to nonexposed plants. Sucrose levels in tubers harvested from 'Norland' and 'Kennebec' plants exposed to ozone were similar to the concentration in the respective control tissue. When means were separated by year fewer significant points were apparent (Figure 2). In 1977 β-glucose levels were significantly higher in tubers harvested from plants of either cultivar exposed to ozone. Tubers from 'Kennebec' plants exposed to ozone also exhibited a significant elevation in total reducing sugar concentration. No significant effects of

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Figure 1. Mean number and weight, and total solids content of potato tubers harvested from 'Norland' and 'Kennebec' plants exposed to acute intermittant doses of ozone. ($\bar{P}$ value signifies probability that values are not significantly different.)

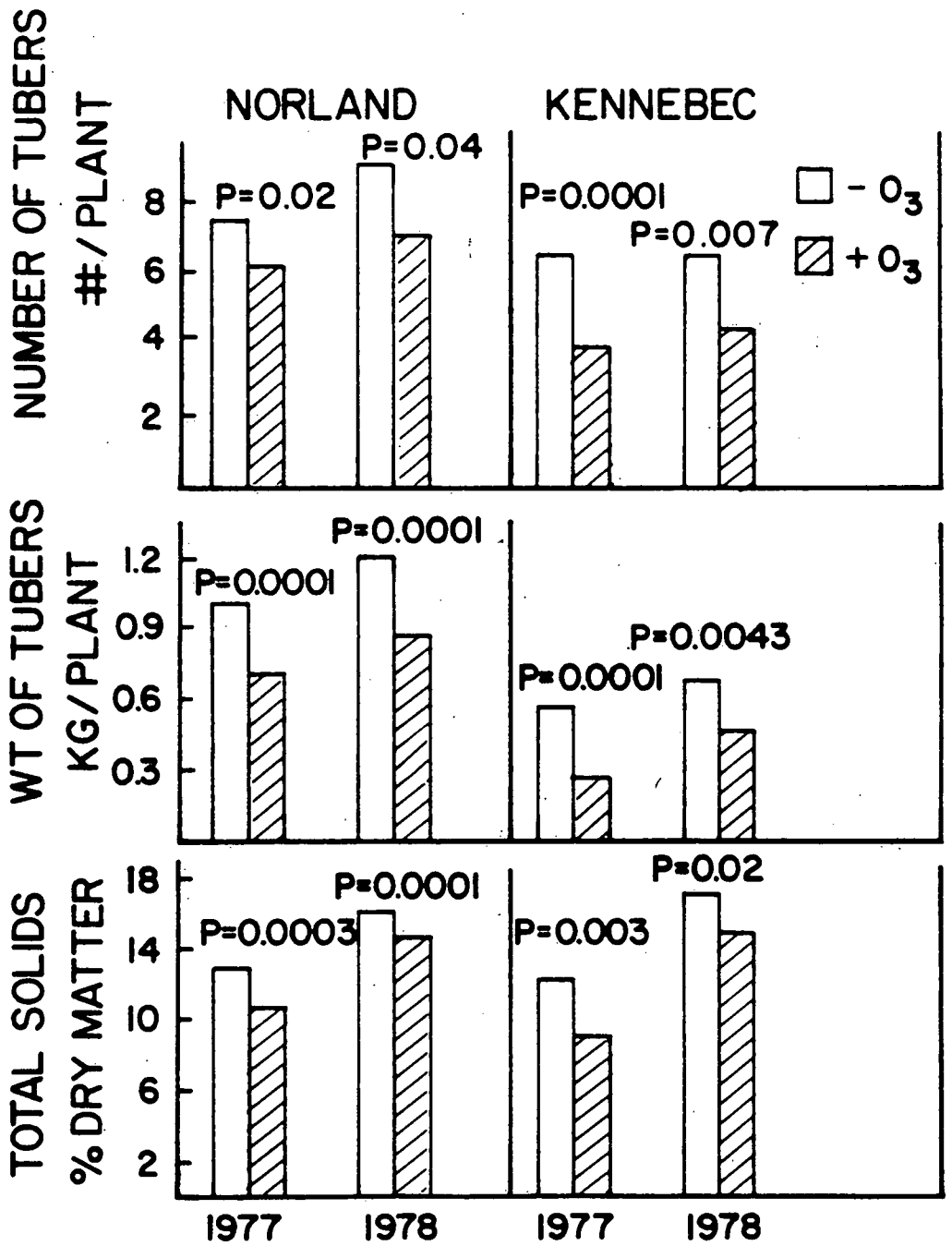
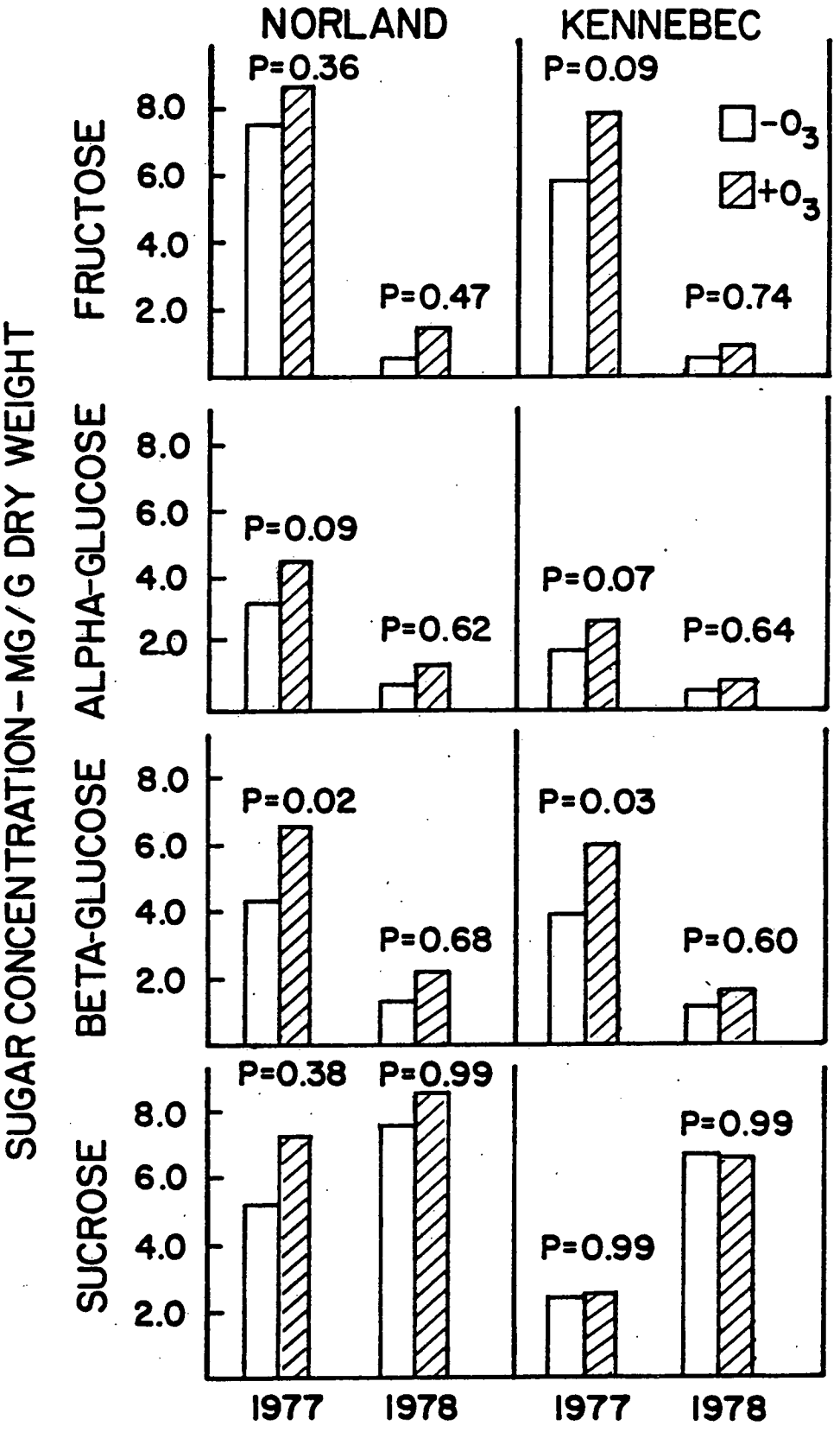

NORLAND
KENNEBEC
NUMBER OF TUBERS
#/PLANT
P=0.02
P=0.04
P=0.0001
P=0.007
□ - O3
▨ + O3
8
6
4
2
WT OF TUBERS
KG/PLANT
P=0.0001
P=0.0001
P=0.0001
P=0.0043
1.2
0.9
0.6
0.3
TOTAL SOLIDS
% DRY MATTER
P=0.0003
P=0.0001
P=0.003
P=0.02
18
14
10
6
2
1977
1978
1977
1978

Figure 2. Mean concentrations of sugars of potato tubers harvested from 'Norland' and 'Kennebec' plants exposed to acute intermittant doses of ozone. (P value signifies probability that values are not significantly different.)


NORLAND
KENNEBEC
P=0.36
P=0.09
P=0.47
P=0.74
P=0.09
P=0.62
P=0.07
P=0.64
P=0.02
P=0.68
P=0.03
P=0.60
P=0.38
P=0.99
P=0.99
P=0.99
FRUCTOSE
ALPHA-GLUCOSE
BETA-GLUCOSE
SUCROSE
SUGAR CONCENTRATION-MG/G DRY WEIGHT
8.0
6.0
4.0
2.0
-O3
+O3
1977
1978
1977
1978

ozone on reducing sugars were detected in tubers harvested in 1978. When sugar concentrations were calculated on a fresh weight basis, no significant effects of ozone were detected in tubers from either cultivar.

Effects of Ozone on TGA.--Levels of TGA from ozone treated plants were significantly (P=0.05) lower than those of nontreated plants when values were expressed on a fresh weight basis. On a dry weight basis, these tubers demonstrated no significant differences (P=0.05) in TGA levels (Table 1). When TGA was expressed on a fresh or dry weight basis, no significant effects due to cultivar were detected and 'Norland' and 'Kennebec' responded similarily to treatment as no interactions were detected (P=0.05). Experimental differences (P=0.05) were detected in the TGA dry weight analysis between experiments I and II. The standard size tubers displayed the same responses to ozone treatment, cultivar, and experiment as the above populations.

There was a significant increase (P=0.05) in total protein in tubers harvested from plants of both cultivars exposed to ozone (Table 2). 'Kennebec' tubers contained a mean of 3.17% and 3.51% total protein on a dry weight basis for control and ozonized tissue respectively. Control tubers of 'Norland' contained a mean of 3.55% total protein on a dry weight basis in contrast to 4.13% total protein in tubers from ozonized plants.

The starch analysis has presented many difficulties. We have just now identified the difficulties and should be able to analyze all the tissue prior to May 31, 1980 (the end of the fiscal year). At that time we will provide all starch data accrued.

Table 1. Mean tuber glycoalkaloid values in plants which received 387 $\mu g/m^3$ (0.20 ppm) ozone or charcoal filtered air for 3 hr once every 2 weeks throughout the growing season.[a]

	Cultivar			
	'Kennebec'		'Norland'	
	Treatment[b]			
Experiment	$-O_3$	$+O_3$	$-O_3$	$+O_3$
I	2.72[c] (0.22)[d]	2.60 (0.23)	2.31 (0.18)	2.09 (0.20)
II	2.80 (0.16)	2.37 (0.17)	2.86 (0.18)	2.00 (0.14)

[a] values expressed are the mean of 20 determinations.

[b] when data were pooled across experiments and cultivars, there was a significant difference between treatments ($p = 0.05$) for data expressed on a fresh weight basis; no significant differences existed when data were expressed on a dry weight basis.

[c] values represent mg tuber glycoalkaloid/100 mg fresh weight tuber tissue.

[d] values enclosed in parenthesis represent mg tuber glycoalkaloid/g dry weight tuber tissue.

Table 2. Percent total protein in tubers harvested from 'Norland and 'Kennebec' potato plants exposed to 0.20 ppm ozone for three hr once every 2 weeks for 110 and 130 days, respectively.[a,b]

	'Norland'		'Kennebec'	
	$-O_3$	$+O_3$	$-O_3$	$+O_3$
	3.38	4.00	3.13	3.94
	3.40	4.33	3.28	3.52
	3.63	4.46	3.66	3.68
	3.93	4.12	2.98	3.69
	3.36	4.14	2.77	3.47
	3.37	3.86	3.21	3.39
	3.63	4.46	3.17	3.47
	3.60	3.79	3.30	3.12
	3.58	4.18	3.47	3.29
	3.60	3.98	2.71	-
$\bar{x}$	3.55 *	4.13	3.17 *	3.51

[a] Experiment conducted between March and August, 1978.

[b] Each value denotes percents protein per g dry weight basis for composit sample of tubers for a given plant.

[c] An * denotes significance of the P = 0.05 level based on an unpaired "t" test.

Discussion

The observation of ozone induced reduction in yield of potatoes was consistent with a previous report (20). Since tubers weighing less than 40 g were discounted, it was possible that ozonized plants did not have reduced yield but rather a larger number of "small" tubers. This did not appear to be the case since there were an equivalent number but reduced total weight of "small" tubers harvested from 'Kennebec' plants exposed to ozone in 1978 when compared with control plants.

'Kennebec' plants exposed to ozone sustained greater yield reduction than 'Norland' in both years, yet foliage of 'Kennebec' exhibited less foliar injury than did 'Norland'. It may be possible that foliar injury stimulated new, secondary growth. Perhaps the higher level of injury sustained by leaves of 'Norland' than 'Kennebec' plants was followed by production of more photosynthetic tissue in the former cultivar. Hence, greater yield reduction in 'Kennebec' may have resulted from relatively less productive foliage.

The larger reduction in yield exhibited by ozonized 'Kennebec' plants may result because of a greater responsiveness of this cultivar to stress per se. Growing potato plants in pots in a greenhouse was a stress, as yields of both cultivars were not comparable with those which would be predicted in the field. 'Kennebec' yields were lower than those of 'Norland' in both years in contrast to observations which would be expected in the field.

Ozone induces a reduction in total solids which is an indication of loss in quality. High percent dry matter is directly correlated with superior quality potatoes (26, 47). It would seem that reduction in proteins does not explain the change in total solids.

However, the results of another study will be necessary before conclusions can be drawn. Starch is an important component of total solids. This data is currently being gathered and should be available within 6 months.

Total solids content of tubers were lower for both cultivars in 1977 than in 1978. Heat stress of plants was a more serious factor during the experiments conducted in 1977 (mean greenhouse temperature 24°C) than in 1978 (mean greenhouse temperature 22°C), and elevated temperature may have contributed to reduced total solids content of tubers. Lower total solids content may have been associated with the oven drying technique utilized in the experiment conducted in 1977. Although oven drying is accepted (26), we were concerned that some volatilization of organic matter might have occurred; hence, in 1978 percent dry matter was determined by lyophilizing tissue. While method of analysis and/or growing environment may have influenced absolute amounts of total solids, ozone reduced percent dry matter in both cultivars in 1977 and 1978.

Whether ozone would induce a reduction in total solids in the field must be tested. Clarke et al. (8) reported that the specific gravity in tubers harvested from 'Norchip' plants grown in an oxidant polluted environment was not significantly different from that of plants of the same cultivar protected by N-[2-(2-oxo-1-imidazolidinyl) ethyl]-N-phenylurea (EDU), a chemical antioxidant. Different properties of tubers analyzed from the latter study and our own could be explained by contrasting experimental design. Clarke et al. analyzed tubers harvested from field-grown plants which were exposed to ambient air pollutants. In our experiment, tubers were sampled from greenhouse grown plants which received acute exposures to ozone in controlled environment chambers. The different

cultivar used by Clarke et al., or comparison between chemically treated and nontreated plants rather than plants not exposed to ozone, might also explain dissimilar results.

Reducing sugars were quantified because of the direct correlation between their concentration and potato chip and french fry color (9, 28, 33). If reducing sugars were to exceed 2% on a dry weight basis or 0.5% on a wet weight basis, respectively, color may become too dark to be commercially acceptable for potato chips or french fries (24, 28). In experiments reported here, these levels were never exceeded. However, ozone did cause statistically significant increases in reducing sugar levels when compared to levels in tubers harvested from nonexposed plants. If potatoes had been grown in an environment which favored elevated levels of reducing sugar e.g., low soil fertility (25), it is possible that the additional stress of ozone would have been sufficient to produce a tuber with an excessive reducing sugar content. There has been evidence that time of harvest influences sugar levels in the tuber (24, 33). We selected a chronological date for harvest of each cultivar. It is possible that sequential harvests would have revealed another time at which sugar levels would have been higher, perhaps exceeding acceptable limits in tubers from ozonized plants. Sugar levels could increase during storage; a tuber already exhibiting elevated sugar levels due to ozone stress might produce enough additional sugar to become unacceptable.

The significant increases in reducing sugars reported on a dry weight basis were probably largely a reflection of the decrease in dry matter induced by ozone. Sucrose levels in tubers harvested from ozonized plants did not change when contrasted with tubers of nonexposed plants. These results may be related to the intermittant exposure of plants to acute

dosages of ozone. Following each exposure, recently matured foliage was visibly injured and photosynthesis was probably impaired (22). Reduction in translocation of sucrose from the leaf to the tuber would lead to reduced starch synthesis (34). It is possible that altered equilibrium would favor some starch hydrolysis to form reducing sugars. As new foliage is formed, sucrose transport would resume at a normal rate and starch synthesis would follow. The large reduction in total solids may result from cumulative effects of the bi-weekly ozone exposure which initiated temporary reduction in starch synthesis. The unchanged sucrose levels observed in harvested tubers may reflect the recovery which occurred between ozonizations. The increase in reducing sugars could be associated with the mode of reporting i.e. dry weight basis, but also with starch hydrolysis. Ozone induced a statistically significant (P=0.05) reduction of TGA levels on a fresh weight basis but the absolute reduction was quite small, i.e., an average reduction of 0.35 mg/100g fresh weight (Table 1). The reduced TGA levels may be related to the effect of ozone on maturity of the plant. All plants exposed to ozone exhibited symptoms of premature senescence similar to those described previously (20, 23). Since factors which delay maturity have been associated with elevated TGA levels (45), premature senescence may similarily relate to reduced TGA levels reported here.

Another explanation lies in the inspection of the dry and fresh weight TGA values as listed in Table 1. Sinden and Webb (45) noted an association of TGA measured on a fresh weight basis with that of total solids content. It is reasonable to expect that any factor which affects total solids may also affect TGA when measured on a fresh weight basis. In this study the significant decrease (P=0.05) in the tuber fresh weight TGA from ozonized plants is a reflection of the concommitant decrease (P=0.05) in total

solids of the same tubers. As a result of the variation encountered in the total solids data, dry weight analysis of TGA was calculated from the solids data to obtain a better indication of absolute changes in TGA levels. In this study, the tubers from ozonized and nontreated plants were similar (P=0.05) in TGA content when measured on a dry weight basis in either experiment I or II, even though differences (P=0.05) existed in total solids levels between those experiments. Thus, it appears evident that under the conditions of these experiments, ozone did not trigger an increase in TGA in either 'Kennebec' or 'Norland' tubers.

The TGA values reported in this study are low in comparison to other published values (45). Whether ozone might elevate TGA content to an unacceptable level in tubers exhibiting higher quantities of TGA prior to the air pollution stress will have to be tested.

Associated Studies

1979 Greenhouse Studies

Between May and October, 1979 a third crop of 'Kennebec' and 'Norland' potatoes was grown in the greenhouse and was subjected to intermittant ozone exposures as in previous years. A number of equipment failures precluded use of the data obtained from this harvest. Hence another study is currently being conducted. By adjusting the photoperiod we expect that starch and protein levels of tubes harvested from plants in this study will be comparable with values accrued from tissue harvested in 1978 (Experiment II discussed earlier).

Leaf Glycoalkaloids

Leaf glycoalkaloids (LGA) have been implicated, although not conclusively, in a protective mechanism against microbial and insect attack (11, 16, 44, 50, 54). Any factor which may affect LGA levels may alter

plant resistance to pests. Obviously such a response would indirectly affect food quality. We were interested in determining whether ozone might directly alter glycoalkaloids in the gaseous receptor viz. the leaf.

Cultural Conditions.--'Norland' and 'Kennebec' plants were germinated under conditions similar to those described for experiment I of the tuber studies. In these experiments, each seed piece was planted in a 907 ml plastic container and no pesticides, fertilizer, staking, or pruning were applied or used.

Ozone Exposure.--Five plants from each cultivar were randomly placed in the exposure chamber the night before fumigation. When plants were 18 days old, they were exposed to 488 ug/m^3 ozone (0.25 ppm) for 3 hr. Approximately 6 hr. later, plants were removed from the exposure chamber and returned to the greenhouse randomly assorted with an equal number of nontreated plants. No attempt was made to quantify foliar ozone injury.

Foliar Sampling.--When plants were 21 days old, the apical portions and bottom four leaves from each vine were removed and discarded. All remaining foliage from each plant was collected, major veins were discarded, and the leaves were frozen in liquid nitrogen and stored at -10°C. Each sample was lyophilized and ground through a 40 mm mesh screen. This experiment was repeated three times.

Analysis of LGA.--One foliar sample, 0.25g dry weight, was analyzed per plant by a method developed for this study. (See attached manuscript describing development of the method - Appendix A). Samples were homogenized in an Omni-mixer (Ivan Sorvall Inc., Newtown, Conn. 06470) at setting No. 3 for 3 minutes with 70 ml of 5% aqueous acetic acid. Crude

extract was transferred to a 12.5 cm Buchner funnel fitted with Whatman No. 1 filter paper. The extract was collected in a sidearm flask by vacuum filtration. The Omni-mixer cannister and filter paper residue were washed with 30 ml of 5% acetic acid; the wash was collected in the same sidearm flask making the total volume about 100 ml. The flask was gently heated at 75°C for 5 minutes. After the flask was removed from the heat, 25 ml of 58% aqueous NH_4OH was added to bring the pH to 10 or greater. The flask was placed in an ice bath to rapidly precipitate the glycoalkaloids which were separated from solution by a 40 minute centrifugation at 9,420g. The supernatant was decanted and the centrifuge tube containing the pellet was placed in a 60°C oven overnight to evaporate the ammonia. The next morning, the pellet was resuspended in 25 ml of methanol and sonicated for 5 minutes. The contents of the centrifuge tube were then gravity filtered through Whatman No. 1 paper; the filtrate was collected directly into a 50 ml hydrolysis flask. The solution was taken to dryness by an air stream and the residue was analyzed as per Fitzpatrick et al. (13, 15) excluding the extraction of Wang et al. (59).

Results

No significant (P=0.05) effects were noted between LGA levels in plants exposed to ozone and those not exposed (Table 3). Highly significant (P=0.01) effects were detected in LGA levels between 'Norland' and 'Kennebec' but these cultivars responded similarily to treatment as no interactions were evident.

Ozone injury to foliage had no effect (P=0.05) on the LGA content of either 'Norland' or 'Kennebec' foliage (Table 3), although differences (P=0.01) were detected between both cultivars and experiments. 'Kennebec' consistently had higher levels of LGA than 'Norland' in all experiments.

Table 3. Mean leaf glycoalkaloid values from potato plants exposed to 488 $\mu g/m^3$ (0.25 ppm) ozone for 3 hr.[a]

	Cultivar[b]			
	'Kennebec'		'Norland'	
	Treatment[d]			
Experiment[c]	$-O_3$	$+O_3$	$-O_3$	$+O_3$
I	3.81	5.23	0.73	0.38
II	5.38	4.89	2.42	1.64
III	5.44	4.78	2.21	2.72

[a] values represent the mean of 5 determinations and are expressed as mg leaf glycoalkaloid/g dry weight leaf tissue.

[b] when data were pooled across experiments, differences in leaf glycoalkaloids between cultivars were significant (P = 0.01).

[c] when data were pooled across cultivars, leaf glycoalkaloid levels in Experiment I were significantly different from those of Experiment II and III (P = 0.01).

[d] when data were pooled across experiments and cultivars, no significant differences were detected between treatments (P = 0.05).

It would be of value to determine what factor(s) triggered the differences in LGA levels between experiments. However, the deviating results in experiment I cannot be explained at this time. Perhaps some undefined environmental parameter could account for the observed variation. It is important to note here, however, that response to ozone treatment was still consistent, as no experiment x treatment interaction was detected.

It is well documented that TGA levels increase if this organ is mechanically damaged (12, 14). It would seem possible that chemical injury to foliage might also increase LGA levels in these organs. Perhaps changes in LGA levels would have been detected if leaves had been sampled later in the post-ozonization period or if foliage which appeared subsequent to the ozone exposure had been analyzed.

Genetic Study and Associated Field Trials

In 1978 we embarked on a major project to determine the inheritance of ozone resistance in potato. This project is funded through a cooperative regional research project and with the use of facilities provided by the D.O.E. contract. For the latter reason and because some of the results have uses in our food quality evaluations, a brief review of the approach and application is discussed below.

Experimental Plan

Twenty seven cultivars of potato identified as early to mid-maturing (R. Webb, Personal Communication) were screened for relative susceptibility to ozone. Based on their differential ozone response, we selected 14 cultivars for use as parents in a breeding scheme. Two 7x7 diallels were constructed and all possible crosses were attempted.

In the spring of 1979 seeds obtained from the crosses were permitted to germinate. Sixty seedlings of each family were planted at Rock Springs Plant Pathology farm (research farm - west of State College, PA) on June 6, 1979. Vines were pulled in late August and tubers were collected from each plant representing a distinct genotype.

After a 4 month dormancy period the progeny of each diallel will be evaluated. Three experiments will be conducted to study each diallel. In every experiment 12 plants of each of the following groups - six parent clones, six self pollinated parents, 15 hybrids, and 15 reciprocals - will be exposed to a fixed dose of ozone.

Methods of data analysis are currently being considered. Data from each experiment within a diallel will be pooled in a combined analysis as outlined by Matzinger et al. (31). In addition, the data will be reexamined in terms of various autotetrapolid genetic models.

Results

The differential susceptibility of the 27 cultivars is indicated on Table 4. The 14 cultivars selected for the diallels are so designated. Diallel I yielded 42 hybrids and diallel II 34 hybrids (Table 4). Successful production of tubers of these hybrid families has been accomplished. Tubers are presently in dormancy awaiting the next phase of this research.

Discussion

This study will provide data which through analysis will help substantiate the heritable nature of ozone resistance in potato and to determine its inheritance by estimation of general and specific combining abilities.

Table 4. Relative ozone susceptibility of 27 potato cultivars employed in an attempt to produce two diallel sets of crosses.

Rank[a]	Diallel I	Diallel II	Rank[a]	Diallel I	Diallel II
1		Norgold Rsst.	15	Norland*	
2	Superior*		16		Peconic*
3		Red LaSoda	17	Monona*	
4	Norchip*		18	Haig*	
5	Chieftain*		19		Manota*
6		Pungo*	20		Rushmore
7		Seminole*	21		Warba
8	Triumph		22		Dazoc*
9		Alamo*	23	Red Warba	
10	Chippewa		24		Waseca
11	I. Cobbler		25	Cherokee*	
12		Blanca*	26	Bake King	
13		Pioneer	27		Mesaba
14		Yampa			

[a] Cultivars listed in order of increasing ozone susceptibility as determined by chamber study.

* Indicates cultivars included among the 7 parents in their respective diallels.

This study is also providing a well defined germ plasm. This germ plasm will be used in the future to more clearly understand the genetics of air pollution tolerance under a diversity of conditions. Furthermore, this germ plasm - the parents and hybrids - will have an application in our food quality trials. Below is a brief discussion of a preliminary field study which we have conducted with an eye toward wedding our projects.

Preliminary Field Studies

The screening of parent cultivars was conducted by assessing injury to small, container-grown plants following exposure to pure ozone under controlled environmental conditions. It is important to know how this method relates to field conditions where more mature plants receive repeated exposures to a diversity of stresses.

We selected East Brunswick, N.J. as a study area because of acknowledged frequency of air pollution stress. Twenty six of the cultivars, replicated 6 times in a randomized complete block design, were planted in a 0.5 acre plot provided by Rutgers University. Each time an inversion resulting in foliar injury occurred, we were notified and we traveled to East Brunswick where injury was assessed.

Because we were interested in tuber quality we planned to analyze sugar, starch and protein content of tubers from four cultivars. We selected 'Cherokee' and 'Norland', and 'Norchip' and 'Superior' based on differential susceptibility and tolerance, respectively. These four cultivars were also grown at the Pennsylvania State University research farm in Rock Springs, an area thought to be less stressed by air pollutants than the New Jersey location. Hourly ozone levels were monitored throughout the summer with a Rem chemiluminescent ozone analyzer.

At harvest, samples of each cultivar were collected. At each of three replicate plots, eight plants were harvested and yield determined per cultivar; all tubers within a cultivar were pooled. Thus, for each cultivar there were three samples per location. The tubers were diced; two 200g subsamples were removed for sugar quantification as previously described. An additional five 100g subsamples were removed and lyophilized. From the latter subsamples, dry weights will be determined and total solids calculated. Total starch and protein analyses will be conducted on these samples.

Results

Air monitoring in New Jersey revealed that ozone levels exceeded the National Air Quality Standard of 0.12 ppm for one hour 30 times between June 1 & August 24, 1979. Many of the potato cultivars planted exhibited foliar injury subsequent to a period of prolonged ozone exposure. The differential cultivar susceptibility observed in the chamber was different than that in the field. However, similarities did exist. 'Superior' and 'Norchip' remained in the tolerant and moderately tolerant categories, respectively. 'Cherokee' and 'Norland' retained their more susceptible status in the field. At Rock Springs, PA, the National Air Quality Standard for ozone was exceeded twice between July 19 and September. The old standard of 0.08 ppm ozone for one hour was exceeded 205 times. While all 27 cultivars were not evaluated for ozone induced foliar injury, spot checks revealed that some injury was present. 'Norland' were quite severly injured and 'Cherokee' exhibited some injury. 'Norchip' and 'Superior' were tolerant.

Yield data for four cultivars considered varied between locations (Table 5). Qualitative analyses have not yet been performed.

Table 5. Mean number and weight of tubers harvested from four cultivars grown in East Brunswick, N. J. and Rock Springs, Pa.[a]

Cultivar	Number		Weight (g)	
	N.J.	Pa.	N.J.	Pa.
Norchip	40.7	50.3	907.0	558.2
Superior	30.7	37.7	750.9	587.0
Cherokee	44.0	26.0	857.3	274.6
Norland	43.7	39.7	816.2	639.4

[a] Each figure represents the mean value of 24 plants, eight harvested from each of 3 plots.

Discussion

These studies are largely preliminary and incomplete. They do, however, give useful information. First, we have further verified the susceptibility of potato to ozone and the presence of frequent elevated levels of ozone in the field. The screening of 27 cultivars in the chamber will be repeated several times this winter. Until that time discussion of the difference in response of the cultivars in the field and chamber would be premature. We should point out, however, that many differences between field and greenhouse grown-chamber exposed plants preclude the likelihood of identical results. Such differences include field use of pesticides, all edaphic factors (soil type, moisture etc.), and air quality, including dosage and spectrum of pollutants present.

It was valuable to confirm that cultivars identified as tolerant and susceptible in chamber studies were indeed responding similarly in the field. We have identified suitable cultivars for future studies. The yield data is minimal and hence preliminary. Obviously, cultivars vary from location to location and differential performance between cultivars varies when contrasted between locations. Also, other factors than ozone influence performance of cultivars more or in conjunction with the air pollutant stress. A few observations are worth noting. In N.J. where the ozone stress was high, 'Norland' and 'Cherokee' produced a greater number of tubers than 'Superior' and 'Norchip'. Whether stressing the foliage might stimulate tuber production is an interesting hypothesis. The increased tuber number observed in plants with ozone

susceptible foliage was not consistent with changes in wet weights of these plant organs. 'Superior' was the worst producer and 'Norland' the best when yield was measured on a wet weight basis. Apparently, some factor other than ozone tolerance was limiting to 'Superior'. When the qualitative data has all been analyzed we will be in a position to suggest more appropriate experiments to determine whether ozone alters quality of potatoes in the field.

II. Alfalfa

There have been a number of published reports identifying alfalfa plants as ozone susceptible. That ozone susceptibility is manifested in appearance of foliar lesions and reduced yield has been documented (37). There is a report of ozone induced reduction in crude fiber, beta-carotene and vitamin C, and increase in niacin (52). Protein efficiency ratio in rats and nitrogen digestibility ratios were unchanged.

The two qualitative factors which we are evaluating are (i) isoflavonoid status and (ii) protein quality.

Isoflavonoid Status

Under certain conditions alfalfa foliage produce elevated levels of isoflavonoids. Some of these compounds have been defined as natural plant estrogens and in elevated concentrations have been associated with aberrant hormonal behavior including infertility in live stock (1, 5, 7, 29, 53).

The hypothesis that ozone would induce isoflavonoids was based on two lines of evidence. Stresses including location, year stage of growth, cutting and biotic disease, all could stimulate production of coumestrol, the most significant of the estrogenic isoflavones (18, 36). In addition, ozone did induce production of three isoflavones viz. coumestrol, daidzein and sojagol in soybean foliage (27).

In the studies conducted in our laboratories we investigated the possibility that ozone might induce estrogenic isoflavones in alfalfa.

Materials and Methods

Plant Culture

Seeds of *Medicago sativa* L. (cultivars will be identified later) were sterilized and planted in Jiffy mix (E. C. Geiger, Box 285, Harleysville, PA) after thorough leaching of the growth medium. Seeds were either planted in 7.6 cm plastic pots (experiment 1) or thereafter in flats containing 96 equally spaced seedlings. Plants were grown in a greenhouse with supplemental incandescent and fluorescent lighting of 4 Klux and a 15 hr. photoperiod in the winter months. Plants were fertilized with 3.6 ml per 2 liters of Peters Peat-Lite Special (Robert B. Peters Co., Inc., 2833 Pennsylvania Street, Allentown, PA) once a week. Insects were controlled with a weekly spraying of Malathion 5E-55 Ortho Chevron or Resmethrin (SBP-1382 EC) (E. C. Geiger, Inc.).

Ozone Exposure

Plants were exposed to ozone by the same methods as have already been described for potato. Concentrations of ozone varied for each experiment.

Experiment One

'Buffalo', an eastern cultivar, was selected to pursue the question of whether ozone could induce production of coumestrol in alfalfa foliage. Two approaches were taken to detect potential induction of coumestrol by ozone. In the first study intensity of the ozone symptom was the variable considered and in the second study time after exposure was considered.

Symptom Severity Study

This study was conducted four times: trials I and II were completed in December and January, respectively, and trials III and IV were completed in August. In trials I, II, and III, 7-wk-old plants were exposed to 387 $\mu g/m^3$ (0.20 ppm) ozone for 2.5 hr. In trial IV plants were exposed to 677 $\mu g/m^3$ (0.35 ppm) ozone for 3 hr. In the four trials, 117, 98, 100, and 100 plants were ozonized, respectively. There were 30 nonozonized control plants in trials I, II, and III, and 35 in trial IV. The foliar symptoms were rated 48 hr. after exposure as follows: 1 = no visible injury; 2 = slight stipple on adaxial leaf surface; 3 = chlorosis and/or necrosis; and 4 = extensive necrosis. Only the most severely injured leaves of each plant were harvested and all leaves of a similar rating were pooled for tissue analysis. In trials I and II, samples were taken from the injury severity classes 1 through 4. In trial III samples from classes 1 through 3 were used, whereas only samples from class 4 were used in trial IV.

Time Study.--Trials I, II, and III were completed in January, February, and July, respectively. When plants were 8.5-wk-old, 30, 72, and 40 plants, in trials I, II, and III, respectively, were exposed to 580 $\mu g/m^3$ (0.30 ppm) ozone for 2 hr. For each trial, 30 plants were maintained as controls. Immediately and 24 hr. after ozone exposure, all middle-aged leaf lamina were harvested from the center one-third of each shoot and were pooled.

Tissue Extraction.--Replicated 1g (fresh weight) samples of pooled foliar tissue were cut into small pieces and placed in vials with 15 ml of 80% methanol. After 3 or more days, the samples were filtered and

washed. The combined filtrate was reduced in vacuo at 40° C to near dryness, then brought to 2 ml in 80% methanol as previously described (42). This solution is referred to as the crude extract. Additional 1-g samples from both studies were used to determine percent dry weight.

Detection of Coumestrol.--Ten-microliter portions of crude extracts were spotted on silica gel G plates, 0.75 mm thick, along with samples of authentic coumestrol (0.04 g/ 1). The plates were developed in hexanes: ethyl acetate:methanol (60:40:1 v/v) and viewed under long-wave UV light.

While no coumestrol was detected, other fluorescent compounds were induced. The one which appeared to be most prominent was the subject of further investigation.

Identification of 4',7-DHF.--To identify 4',7-DHF, silica gel G plates, 1.0 mm thick, were streaked with 0.5-2.0 ml of crude extract and developed in toluene§ethyl formate:formic acid (5:4:1). The bright whitish blue fluorescent band was scraped from the plates, eluted with methanol, dried, and redissolved in 8 or 18 ml of methanol. A second purification was performed by descending chromatography on Whatman 3 MM paper in 30% acetic acid. After drying for about 18 hr. the fluorescent band was eluted for 10 min. in spectroscopic grade methanol. The solution was filtered and centrifuged to remove paper particles, and analyzed immediately with a Gilford 2400-S spectrophotometer (Gilford Instrument, Inc., Oberlin, OH 44074). Spectra in methanol, supplemented with five reagents, were obtained as described by Mabry et al. (30). Spectra of authentic 4',7-DHF (provided by A. L. Livingston and B. E. Knuckles, USDA, Western Regional Research Laboratory, Albany, CA 94706) also were obtained. The purified methanol solution of the alfalfa compound and authentic 4',7-DHF were

compared by silica gel thin-layer chromatography (TLC) and by two-dimensional paper chromatography (2-D PC). Solvents for TLC included: hexanes:ethyl acetate:methanol (60:40:1), benzene:ethyl acetate (3:1), toluene:ethyl formate:formic acid (5:4:1), and chloroform:methanol (3:1). Solvents for 2-D PC were tertiary butanol:acetic acid:water (3:1:1) and 15% acetic acid. After development of the paper chromatograms, the color of fluorescence of the compound was noted before and after exposure to ammonia vapors.

Quantification of 4',7-DHF.--Relative concentrations of 4',7-DHF were estimated by fluorometry. Ten-microliter aliquots of crude extracts were spotted on silica gel plates, 0.75 mm thick. The plates were developed in hexanes:ethyl acetate:methanol (60:40:1). Relative fluorescence of the 4',7-DHF spots was measured with a photofluorometer as described by Bailey (4), except that we used a Photovolt Model 520-M Photometer (Photovolt Inc., New York, NY 10010) with a 2-mm aperture. From 2-13 samples (usually 4-7 samples) were tested for each treatment by trial combination. Data were analyzed by one-way and two-way analyses of variance. Square root transformations of the data were necessary because data were nonlinear.

During the latter part of the study, when identification of 4',7-DHF was completed and authentic material was available, TLC plates were spotted with known amounts of authentic material as well as with crude extract samples, to estimate actual amounts of 4',7-DHF in the samples. The minimum level of detection was 2 µg/g dry wt. A galvanometer reading of 0.5 indicated 11.6 µg of 4',7-DHF per gram of tissue (dry wt), and a reading of 53.5 indicated about 1,370 µg/g. The relationship of galvanometer deflection to actual amounts of compound was not linear.

Experiment Two

After we determined that coumestrol was not produced in 'Buffalo' in response to ozone, we considered it important to discern whether this "null" response was similar for other cultivars of alfalfa. It was also of interest to determine whether the ability of ozone to stimulate increases in concentrations of other flavonoids was genetically determined.

Medicago sativa L. 'Moapa', 'Sonora', 'Ladak', and Medicago sativa L. x Medicago falcata L. 'Vernal' were selected for the study. In November and December, 1977, 7-week-old seedlings were exposed to 773 $\mu g/m^3$ (0.40 ppm) ozone for 3 hr. as previously described. The experiment was repeated 3 times. Seven flats of alfalfa were exposed to ozone and three flats were maintained as controls; each flat contained 24 plants of each cultivar.

Leaves were harvested 48 hours after ozone exposure. Since we had established that maximum flavonoid induction occurred in foliage exhibiting symptoms which ranged from chlorosis to extensive necrosis, all leaves within a cultivar that exhibited these symptoms were harvested and pooled. All middle-aged leaves of control plants, similar in developmental stage to injured tissue, were also harvested. From each sample per cultivar per trial, four 1g subsamples were cut into small pieces and extracted for 3 days or longer in 15 ml of 80% methanol. An additional set of ten 1g subsamples per cultivar per trial were taken for determination of dry weight. Additional extraction procedures followed methods described above.

Detection and quantitation of coumestrol and 4',7-DHF were accomplished by thin layer chromatography and fluorometry according to the protocol previously specified. Our method varied from that used in

experiment I in the use of 0.25-mm-thick Silica Gel G (Applied Sciences Lab., State College, PA 16801) plates. A standard curve was prepared for 4',7-DHF by quantifying fluorescence of 14 spots of the authentic compound (provided by A. L. Livingston and B. E. Knuckles, USDA, Western Regional Res. Lab., Albany, CA 94706) at concentrations ranging from 0.01 to 0.25 g. Concentrations of 4',7-DHF in samples were determined from the standard curves and reported as ppm on a dry weight basis. Differences in levels of 4',7-DHF induced in the four cultivars were tested by analysis of variance.

Experiment Three

In a third experiment 'Moapa', one of the most susceptible of the cultivars studied, and one of the most vigorous, was selected. In this series of studies the ability of ozone to induce formononetin, daidzein, and genistein was considered.

Alfalfa seedlings were cultured by methods already described. Six to seven-week-old plants were placed in a modified Environmental Growth Chamber 15-20 hr. before fumigation. Plants were exposed to 580 ug/m (0.30 ppm) ozone for two hours beginning at 10:00 AM; flats were returned to the greenhouse 3-4 hr. after fumigation. Three ozone exposures were conducted with three flats of alfalfa seedlings per fumigation. Nonexposed plants of the same age were maintained in the greenhouse.

Extraction of Isoflavones

Forty-eight hours after ozone exposure, all middle-aged leaves which exhibited ozone induced chlorosis and/or necrosis were harvested. All corresponding middle-aged leaves of the nonexposed plants were also harvested.

All leaves within a treatment were pooled and mixed, and 3 g subsamples were removed. Each subsample was placed in a mortar and pestle and ground in liquid nitrogen (36). The frozen material was then placed in 50 ml boiling 90% methanol for 1 min. The beakers were covered with Parafilm and stored in the dark at room temperature for a minimum of three days. The methanol extract was vacuum filtered through two layers of Whatman No. 1 filter paper in a Buchner funnel and rinsed with 15 ml 100% methanol. The combined filtrate was flash evaporated to near dryness under reduced pressure at 50° C and the residue was brought to a volume of 2 ml with methanol. These samples of crude extract were stored at 4° C.

Detection of Isoflavones

Two-dimensional paper chromatography (2-DPC) was employed to screen the crude extract for presence of isoflavones. Two hundred to 500 ul of crude extract were spotted in the lower left corner of Whatman 3 MM paper. Twenty-five to 50 ul of authentic samples (approximately 0.025 mg/ml) of formononetin, daidzein and genistein were spotted in the margins of the papers to determine approximate positions within crude extract.

The chromatograms were developed descending in the first dimension in the solvent system of tertiary-butyl alcohol:acetic acid:water (3:1:1) and in the second dimension in 15% acetic acid in water (30). In order to

obtain a better separation of the isoflavones, other chromatograms were developed descending in benzene:acetic acid:water (125:72:3) for the first dimension and in 2N ammonia for the second dimension (60). The papers were then viewed under short wave ultraviolet light (UV - 254 nm) with and without fuming with ammonia vapors. Color and Rf values of spots were noted.

Detection of the presence of daidzein, genistein, and formonetin was further investigated by spotting 20 ul of crude extract and 10 ul of each of the isoflavones on 0.25 mm silica gel on thin layer plates. Plates were developed in a chloroform:methanol (95:5) solvent system with which we were able to achieve successful separation of the three authentic compounds.

The plates were viewed under UV light with and without prior fuming with ammonia vapors. In order to facilitate viewing genistein, plates were sprayed with diazotized sulfanilic acid and then fumed with ammonia (41). The Rf values were determined for the authentic compounds in each solvent system and color of spots was noted.

Purification and Identification of Unknowns

The unknown compounds were purified as follows. Thin layer silica gel plates (20 x 20 cm) were streaked with 500 ul crude extract alone with 10 ul of the authentic compounds spotted at the margins of the plate. Plates were developed for 60 min. in chloroform:methanol and allowed to dry for 2 hr. under a hood. The fluorescent bands of unknown material, which closely corresponded with the authentic compounds in position and color, were scraped and eluted in 50 ml of methanol for 8-10 min. The solutions were filtered through a fritted glass funnel with one layer of Whatman No. 1 filter paper and washed

with a few milliliters of methanol. Each eluate was flash evaporated at 50°C to dryness and then dissolved with 2 ml methanol.

Solutions of partly pure unknowns were spotted on fluorescent silica gel plates (.25 mm, SIL-G-25 UV254) and developed in the chloroform-methanol solvent system in order to determine the homogeneity of the partly pure solution.

In an attempt to separate the compounds and isolate the one suspected of being formononetin, the partly pure preparation was subjected to descending paper chromatography in two successive systems. One to two milliliters of partly pure extract, described above, were streaked on Whatman 3 MM papers. The papers were developed descending using 30% acetic acid in water (36) for 6-7 hr., then dried in a fume hood overnight. The dried paper was viewed under UV light. The strip, bearing a compound with a similar Rf value and fluorescence to authentic formononetin, was cut into small pieces, eluted in 50 ml spectral grade methanol for 8-10 min. with intermittant swirling, and filtered through a fritted glass funnel with one layer of Whatman No. 1 filter paper. Control samples consisting of blank papers were developed and eluted at a similar position and by the method as just described for the unknown. The filtrates from both unknown and blank papers were flash evaporated to dryness and the residues redissolved in 10 ml and 5 ml, respectively, spectral grade methanol. Purified extracts were scanned from 200 - 350 nm with a Beckman Model 3600 Spectrophotometer. The spectrum of authentic formononetin in methanol was obtained for comparison with that of the unknown.

The partly pure eluate was then streaked on chromatographic paper and developed with tert-butyl alcohol:acetic acid:water (3:1:1) in order to obtain a more purified compound. A blank paper was also developed in this solvent system. The band coinciding with formononetin was then eluted and evaluated by ultraviolet spectroscopy as described above.

Since the eluate purified by 2-DPC did not provide a similar UV spectrograph to authentic formononetin, thin layer plates were again streaked with a more concentrated crude extract prepared from three 5 g subsamples of ozonized leaf tissue and developed in the chloroform: methanol system to partly purify the unknown compound. Two bands were observed that corresponded closely to the position of the authentic when viewed under short wave UV light. One of the bands and the authentic formononetin disappeared under long wave UV; this unknown band was analyzed by ultraviolet spectroscopy immediately after TLC.

Two dimensional thin layer co-chromatography was also conducted to determine if several compounds were present in this partly pure extract. Fluorescent TLC plates were spotted with either 250 ul of the partly pure unknown, a mixture of 250 ul of partly pure compound and 10 ul of the authentic alone. The plates were developed in the first dimension with chloroform:methanol (95:5) and in the second dimension with benzene:ethyl acetate (80:20).

Results

Experiment One

Symptoms.--Injured leaves exhibited chlorosis or slight necrosis 24 hr. after exposure and later more extended necrosis developed. The pattern of injury varied, sometimes occurring on the leaf margins and interveinal areas of small, isolated patches and other times only

on tissue adjacent to the veins. Slight injury appeared as a faint, chlorotic stipple on the adaxial surface. Dark, watersoaked spots, seen immediately after ozone exposure, often occurred on foliage on which more severe injury developed later.

In the symptom severity study, percentage of leaves in each injury class varied among plants harvested 48 hr. after ozone exposure, but the distributions for trials I, II, and IV were generally similar (Table 6). The foliage from plants in the first two trials, completed in December and January, exhibited 87% visible injury (injury classes 2-4). In trial III, completed in August, only 44% of the foliage was visibly injured. Plants in trial IV, also conducted in August but with a higher dosage of ozone, had 79% visibly injured leaves.

In trials I and II of the time study, conducted in January and February, 67% and 81% of the leaves, respectively, were visibly injured (Table 6). Only 46% of the leaves in trial III, conducted in July, showed visible symptoms.

Identification of Compounds.--Coumestrol was not detected in any sample (control or experimental) from either experiment. The minimum level of detection of coumestrol was 2 μg/g dry wt.

Fluorescence on 2-DPC of ozone-injured leaf samples appeared in seven different areas but was not present in crude extract samples from healthy foliage. The most conspicuous whitish blue fluorescent area was identified as 4',7-DHF. When the compound was exposed to ammonia vapors, there was a color change to yellow-green fluorescence under UV light, as reported previously (6, 30). The compound extracted from alfalfa gave UV spectra in six reagents that were identical to published spectra (30) and to spectra obtained from an authentic sample. The unknown compound and authentic

Table 6. Percent of alfalfa leaves showing different symptoms of ozone injury in symptom severity and time study trials.

Symptoms	Symptom severity study trials[a]				Time study trials[b]		
	I Dec. (%)	II Jan. (%)	III Aug. (%)	IV Aug. (%)	I Jan. (%)	II Feb. (%)	III Aug. (%)
No visible injury	13	13	56	21	33	19	54
Slight stipple	39	18	30	32	29	28	32
Chlorosis and/or necrosis	30	28	12	22	28	28	11
Extensive necrosis	18	41	2	25	10	25	3

[a] Plants were exposed to 387 $\mu g/m^3$ (0.20 ppm) ozone for 2.5 hr, but plants in trial IV were exposed to 677 $\mu g/m^3$ (0.35 ppm) ozone for 3 hr. Samples were harvested for tissue analysis and symptoms were rated 48 hr after exposure.

[b] Plants were exposed to 580 $\mu g/m^3$ (0.30 ppm) ozone for 2 hr. Samples were harvested at 0 and 24 hr for tissue analysis; symptoms were rated 24 hr after exposure.

4',7-DHF had identical Rf values after silica gel TLC with all solvent systems used: hexanes:ethyl acetate:methanol, Rf = 0.08; benzene:ethyl acetate, Rf = 0.07; toluene:ethyl formate:formic acid, Rf = 0.52; and chloroform: methanol, Rf = 0.85. With 2-DPC, Rf values were 0.83 and 0.09 in tertiary-butyl alcohol:acetic acid:water, and 15% acetic acid, respectively. After PC in 30% acetic acid, Rf was 0.42. This compared favorably with other reports of 0.40 and 0.37 (36,61).

Concentration of 4',7-DHF.--Both symptom severity and date of the trial affected the concentration of 4',7-DHF (Table 7). Two-way analysis of variance of the square roots of individual observations showed that both factors were significant (5% level). Nonozonized controls and ozonized leaves showing no visible injury had either no 4',7-DHF or relatively low levels. Leaves ozonized in December or January, and showing chlorosis or necrosis, had relatively high levels. Concentrations were highest in severely injured leaves; one sample had approximately 1,370 µg of the flavone per gram of tissue. The leaves treated in August had only low levels of the flavone, however, regardless of symptom severity. In a second trial in August, in which plants were exposed to 0.35 ppm ozone for 3 hr. to induce extensive necrosis (Table 6), 4',7-DHF was relatively low in severely injured leaves.

In the time study, nonozonized controls contained no detectable 4',7-DHF in January and August and low levels of the flavone in February (Table 8). Ozonized plants also had little or no 4',7-DHF immediately after exposure. In August, a relatively low level of

Table 7. Relative fluorescence of 4',7-dihydroxyflavone extracted from alfalfa leaves showing different symptom severity 48 hr after 2.5 hr exposure to 387 μg/m^3 (0.20 ppm) ozone.

Symptoms	Trials[a] I Dec.	II Jan.	III Aug.
Control (no ozone)	1.7[a](5)[b]	5.1 (5)	0.0 (7)
No visible injury	0.0 (3)	6.5 (3)	1.0 (7)
Slight stipple	5.8 (4)	12.5 (2)	1.7 (6)
Chlorosis and/or necrosis	27.6 (4)	32.5 (5)	1.5 (4)
Extensive necrosis	46.5 (2)	37.0 (3)	- [c]
Standard deviation of means[d]	5.6	2.4	0.6

[a] Galvanometer deflections produced by fluorescent areas on thin layer chromatography plates and quantified with a photometer. Deflection of zero indicates concentration below 2 μg/g (dry wt) or nil. Deflection of 12.5 indicates approximately 100 μg/g of flavone.

[b] Number of samples per value is indicated in parentheses.

[c] Insufficient material for assay.

[d] Pooled standard deviations within the trial.

Table 8. Relative fluorescence of 4',7-dihydroxyflavone from alfalfa leaves harvested 0 and 24 hr after 2-hr exposure to 580µg/m^3 (0.30 ppm) ozone.

Treatment	Trials I Jan.	II Feb.	III Aug.
Control			
0 hr	0[a] (4)[b]	4.8 (5)	0 (7)
24 hr	–[c]	4.6 (9)	0 (7)
Ozone			
0 hr	1.2 (7)	5.6 (7)	0 (7)
24 hr	15.4 (13)	27.9 (9)	1 (7)
Standard deviation of means[d]	2.6	3.3	0.2

[a] Galvanometer deflections produced by fluorescent areas on thin layer chromatography plates and quantified with a photometer. Deflection of zero indicates concentration below 2 µg/g (dry wt) or nil. Deflection of 15.4 indicates approximately 110 µg/g.

[b] Number of samples per value is indicated in parentheses.

[c] Insufficient material for assay.

[d] Pooled standard deviation within the trial.

4',7-DHF was detected 24 hr. after exposure, but in January and February there was a significant increase 24 hr. after exposure, compared with content immediately after exposure.

Experiment Two

Visible injury was not apparent on all plants following exposure to ozone. Symptoms occurred either on the leaf margins and interveinal areas of middle-aged foliage in small isolated patches or on foliar tissue directly adjacent to the veins.

Coumestrol was never detected in tissue from control or ozone-treated plants. 4',7-dihydroxyflavone was induced by ozone in foliage of all four alfalfa cultivars (Table 9) and there were no significant differences in concentrations of 4',7-DHF among the four cultivars. The highest level of 4',7-DHF in an individual sample was 91 ppm in the cultivar 'Ladak.' No. 4',7-DHF was detected in control tissue of any cultivars with a minimum level of detection of 2 ppm (dry wt. basis).

Experiment Three

Detection of Isoflavones

Twenty fluorescent areas were observed when the crude extract from the ozonized tissue was subjected to 2-DPC. The benzene:acetic acid:water and 2N ammonia solvent system provided better separation of the isoflavonoid components of the crude extract than did the tertiary-butyl alcohol:acetic acid:water and 15% acetic acid in water solvent systems. Three spots were observed which were similar to daidzein, formononetin, and genistein in color and position.

Authentic compounds on thin layer plates separated readily when plates were developed in chloroform:methanol (95:5). Formononetin and daidzein both exhibited a bright, light-blue color under UV light

Table 9.. Occurrence of 4',7-DHF in foliage from four cultivars of alfalfa, 48 hours after exposure to 773 $\mu g/m^3$ (0.40 ppm) ozone for 3 hours.[a]

Cultivar	Experiment[b] I	II	III	Mean
	ppm dry wt			
'Ladak'	60 ± 16[c]	50 ± 19	42 ± 17	51
'Sonora'	41 ± 16	64 ± 28	35 ± 11	47
'Vernal'	43 ± 18	47 ± 3	46 ± 19	45
'Moapa'	44 ± 20	53 ± 15	47 ± 20	48

[a] Control tissue was analyzed in conjunction with ozonized tissue 4',7-DHF, if present, was <2 ppm (the detection limit) in control tissue.

[b] Each value is the mean of four samples of tissue analyzed. Tests for equality of means for each cultivar and experiment are based on an analysis of variance (P = 0.05).

[c] Standard deviation.

which became brighter after fuming with ammonia. Genistein exhibited a dark purple color with and without fuming with ammonia and turned an orange-brown color when sprayed with diazotized sulphanilic acid and fumed with ammonia.

Seven to eight fluorescent spots were observed when the crude extract of leaves exposed to ozone was spotted on thin layer plates and developed in the chloroform:methanol (95:5) solvent system. Plates streaked with crude extract from nonexposed alfalfa foliage did not exhibit these fluorescent compounds. Fluorescent spots were observed which were similar to the three authentic compounds in color and position.

Purification and Identification of Unknowns

When partly pure unknowns were subjected to TLC no spot on fluorescent plates resembled authentic genistein or daidzein in color or position; these compounds were eliminated from further study. The third unknown separated into three spots, one of which was similar to the authentic formononetin in color and position.

After running the unknown in the 1st dimension of 30% acetic acid in water, eluting, and rerunning the unknown with tertiary-butyl alcohol: acetic acid:water, the methanolic extract of the purified compound was scanned by ultraviolet spectroscopy. The spectrum for the unknown compound, eluted after each run by paper chromatography, was not identical to that of authentic formononetin in methanol.

When thin layer plates were streaked with a concentrated amount of crude extract, it was observed that two fluorescent bands corresponded closely to the Rf of the authentic formononetin. The bands were viewed under both long and short wave UV light. Authentic formononetin and

one band fluoresced more brightly under the short than long wave UV light. The other band was visible under both long and short wave UV light and, therefore, was discounted. The eluate of the band resembling authentic formononetin was analyzed spectrophotometrically. The UV spectrum of this band was dissimilar from that of authentic formononetin but similar to the spectrum of the compound extracted from 2-DPC as discussed above. The absorption maximum was 248 nm for authentic formononetin and 237 nm for the unknown.

When 2-DTLC was conducted on fluorescent plates, the partly purified eluate and the authentic formononetin travelled together when developed in the first dimension of chloroform:methanol with only one spot visible on all three plates. In the second dimension of benzene:ethyl acetate, it was observed that four compounds were present in the unknown solution with compound #3 closely resembling authentic formononetin. The Rf of this compound was similar to authentic formononetin (Table 10). The spot on plate 1, where the unknown was spotted with authentic, was brighter than on plate 2, where unknown was spotted alone.

While ozone does induce 4',7-DHF in injured foliage, its levels do not always increase. Apparently some interacting factor influences the ability of the plant to respond to ozone in this manner.

Discussion

Ozone apparently induces increased concentrations of flavonoid compounds in alfalfa foliage, as many compounds with such properties were revealed in 2-DPC in experiments one and three. One of the compounds was identified as 4',7-dihydroxyflavone. Symptom severity and symptom development appear to correlate with increased levels of these compounds. One might speculate that the unknown biological

Table 10. Rf values of unknown compounds separated from partly purified unknown preparation and of authentic formononetin as determined by two-dimensional silica gel thin layer co-chromatography.

	First Dimension (chloroform-methanol) (95:5)	Second Dimension (benzene-ethyl acetate) (40:10)			
		Compound			
		1	2	3	4
Plate 1					
(unknown + authentic)	.39	.08	.12	.26	.42
Plate 2					
(unknown)	.61	.08	.16	.29	.42
Plate 3					
(authentic)	.55		.31		

significance of 4',7-DHF is associated with cell necrosis. We were able to detect coumestrol in fungally infected alfalfa leaves, hence the absence of the compound was not due to faulty technique. Whether ozone might enhance isoflavonoid production of alfalfa foliage with some fungal infection might be a topic for further study.

None of the estrogenic isoflavonoids are induced in alfalfa in response to ozone, at least not at levels of biological importance. Sherwood et al. (42) have suggested that fungal enzymes are necessary to release the isoflavone from its glycosidic precursor. Perhaps the ability of Keene and Taylor (27) to detect isoflavonoids in soybean is due to the presence of an enzyme in this legume which is absent in alfalfa.

Protein Quality

Alfalfa is one of the most important forage crops grown in the United States. Its value is based on high protein content. Some of the proteins have been characterized with positive and negative attributes. The purpose of this study was to extract foliar proteins, identify and characterize the major bands and contrast their presence and prominence in ozone treated and control tissue.

Methods

Methods of culture and ozone exposure were similar to those already described. 'Moapa' is the cultivar being used in this study.

Methods of extraction, purification by column chromatography and preparation by gel electrophoresis are currently being developed to characterize the protein fraction.

No results are available but we hope to have sufficient data to identify changes in protein quality in response to ozone by the summer of 1980.

III. Soybeans

Materials and Methods

Seeds of glycine max L. 'Chippewa' were cultivated in 10" diameter pots on April 20, 1978 in a 2:1 sand:mushroom composte soil. All seeds were innoculated with Rhizobium japonicum and root nodulation was later verified. Plants were fertilized weekly with a 9:45:15 (NPK) mixture. A systemic insecticide-disyston was applied to the soil initially. We verified that disyston did not influence sensitivity of soybean plants to ozone.

Twenty plants were exposed to 0.25 ppm ozone for 3 hours every second week beginning May 9, 1978. Twenty additional plants were maintained in the greenhouse as controls. In mid July ozone exposures ceased and in mid August pods were harvested and yield quantified.

Total extractable lipid and total protein content was measured for a pooled sample from each plant (3).

Results

Ozone exposure caused a characteristic fleck of susceptible foliage at each fumigation. The symptoms were uniform for all exposed plants. Ozone significantly reduced the weight and number of seeds per plant and number of pods per plant (Table 11). Our results are consistent with published efforts. There were no significant differences in total lipid or protein content when treated and control plants were contrasted.

Table 11. Quantity and quality of soybeans harvested from plants exposed to 0.25 ppm O_3/3hr once every 2 weeks throughout their growth.

	$-O_3$		$+O_3$
Total wt of seeds/plant	28.8[a]	*	18.4
# seeds/plant	164	*	124
# pods/plant	72.5		54.7
# seeds/pod	2.2		2.3
crude protein % (dry wt)	39.94		41.13
other extractable lipid % (dry wt)	20.04		20.67

[a] each value is a mean from ten plants.

* denotes significance at the p=0.05 level based on an unpaired "t" test.

Discussion

Before conclusions can be drawn a second study must be conducted. In 1979 we attempted to repeat the 1978 study. Unusual foliar symptoms present throughout the experiment raised concern about the health of our plants. Through substantial soil and foliar tests, we discovered that our soil was contaminated with zinc. Although this discovery forced us to abort the experiment one observation is worth noting. At each ozone exposure we were struck by the extreme sensitivity of this set of soybeans in contrast to the tolerance of previous sets observed over a six year period in this laboratory. There are several published reports that zinc predisposes plants to ozone (32). Hence, when the soil analysis revealed zinc contamination the explanation for the sensitivity of the plants to ozone became apparent. We tried to grow another set of plants beginning in September, 1979. While we created a photoperiod which matched the April-August photoperiod of the previous experiments all plants grew slowly and senesced prematurely. From previous experience it is apparent that low light intensity and cooler greenhouse temperatures prevent optimal growth of this species. Next April we plan to repeat this study.

It appeared from the first year's data that total lipids were unaffected. However, a gas chromatographic separation of compounds would be worth considering since a change in ratio of individual lipids could have occurred. Changes in randomization of lipids are known to influence melting point of oils (38).

While total protein content in beans of control and treated plants were not statistically different there appeared to be a slight increase in beans from ozone treated plants. Perhaps a different experimental design would have revealed a more significant difference. Consideration of amino acid ratio would be worthwhile. In addition a qualitative evaluation of proteins should be conducted. There are undesirable proteins in soybeans which can be found in elevated concentrations. Tryptin inhibitors have been found in soybeans and these inhibitors have been associated with hypertrophy of the pancreas in swine (Kenneth Wright, personal communication).

The direction which this study takes will await results of the second experiment.

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*Presented at National American Phytopathological Society Meetings.

Non Refereed:

The research has received considerable local attention. Our work has been:

1. featured on a public television program entitled "Weather World";
2. featured in a news story entitled "How Ozone Damages Plants" which has received wide coverage;
3. subject of an article scheduled to be published in an in house organ Science in Agriculture.

In addition to formal presentations of our research as indicated under "abstracts" we have presented our findings in a more informal setting at two national Air Pollution Workshops:

1. Minneapolis, MN. 1978
2. Sudbury, Ontario 1979

APPENDIX

Modified Method for Tuber Glycoalkaloid and Leaf Glycoalkaloid Analysis[1]

J. J. Speroni and E. J. Pell[2]

Abstract

A widely employed, comprehensive method exists for the determination of tuber glycoalkaloids (TGA). This method, which consists of a bisolvent extraction, preparation and purification of aglycones and subsequent titration, was not acceptable for quantification of leaf glycoalkaloids (LGA). A modification of an existing extraction procedure was developed and tested. When the original bisolvent and the newly developed acetic acid extractions were contrasted 1 percent recovery of authentic α-solanine from leaf tissue was 19-27% and 91-96%, respectively. This new procedure was easily adapted for TGA analysis and demonstrated improved recovery of solanine from tuber tissue when compared with the bisolvent extraction.

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TO TYPIST—Begin typing flush with the left-hand marginal line, and end typing so the average length of line corresponds with the right-hand marginal line.

Introduction

In a study reported elsewhere (7), the impact of the air pollutant ozone on LGA levels in the potato was evaluated. The comprehensive method of Fitzpatrick et al. (3, 4) including the Wang et al. (8) bi-solvent extraction for the determination of tuber glycoalkaloids was found to be inadequate for the analysis of leaf glycoalkaloids. It was necessary to develop a more efficient procedure. The method developed was a modification of the established 5% acetic acid extraction combined with the hydrolysis, benzene extraction and titration method as developed by Fitzpatrick et al. (3, 4). This procedure, referred to as the 5% acetic acid extraction, provided a more refined technique for removing glycoalkaloids from foliage and tubers which permitted improved reproducibility and recovery of authentic α-solanine.

Materials and Methods

Analyses were performed on freeze-dried foliar samples of 0.25 g dry weight and unpeeled tuber samples of 20 g fresh weight. Samples were homogenized in an Omni-mixer (Ivan Sorvall Inc., Newtown, Conn. 06470) at setting No. 3 for 3 minutes with 70 ml of 5% aqueous acetic acid. Crude extract was transferred to a 12.5 cm Buchner funnel fitted with Whatman No. 1 filter paper. The extract was collected in a side-arm flask by vacuum filtration. The Omni-mixer cannister and filter paper residue were washed with 30 ml of 5% acetic acid; the wash was collected in the same sidearm flask making the total volume about 100 ml. The flask was gently heated to 75° C, removed from the heat, and 25 ml of 58% aqueous NH_4OH was added to raise the pH to 10 or greater.

The glycoalkaloids were rapidly precipitated in an ice bath and pelleted by centrifugation at 9,420 g for 40 minutes. The supernatant was decanted and the centrifuge tube containing the pellet was placed in a 60° C oven overnight to evaporate the ammonia. The next morning, the pellet was resuspended in 25 ml of methanol and sonicated for 5 minutes. The contents of the centrifuge tube were then gravity filtered through Whatman No. 1 paper; the filtrate was collected directly into a 50 ml hydrolysis flask. The solution was taken to dryness by an air stream and the residue was analyzed as per Fitzpatrick et al. (3, 4) excluding the Wang extraction (8).

Both tuber and foliar tissues were subjected to the bisolvent extraction as well as the 5% acetic acid extraction in order to compare efficiency of the procedures. Glycoalkaloid content and efficiency of recovery of authentic solanine, added at the onset of the procedure, were determined by each method for the respective tissues. To confirm that glycoalkaloids could be recovered by the 5% acetic acid extraction, thin-layer chromatography (TLC) was employed. Samples of 'Kennebec' foliage were subjected to the 5% acetic acid extraction procedure as previously described; after the precipitated glycoalkaloids were resuspended in methanol, the residue was air dried until about 2 or 3 ml remained. Approximately 10 µl of this eluate were spotted on TLC plates (20 cm^2, 0.25 cm thick) precoated with Silica Gel G. Authentic solanine (gift of S. F. Osman and T. J. Fitzpatrick, S.E.A., U.S.D.A., 600 Mermaid Lane, Philadelphia, Pa. 19118) and tomatine (purchased from Sigma Chemical Co., P. O. Box 14508, St. Louis,

Missouri 63178) samples were prepared in methanol (1 µg/ml) and spotted on the plates at volumes of 10 µl each. Plates were developed in the organic layer of chloroform:95% ethanol:1% ammonium hydroxide (2:2:1 V/V) and air dried for at least one hour (1, 6). Plates were fumed with iodine vapors in order to reveal glycoalkaloids (9).

Results

When tuber tissue was analyzed after comparable samples were subjected to each of the extraction procedures only small differences in absolute TGA levels were detected; standard deviations were lower in all samples analyzed with the 5% acetic acid extraction (table 1). When LGA were extracted by the 5% acetic acid method, levels were higher than when extractions were accomplished by the bisolvent procedure. Results of recovery experiments conducted with authentic α-solanine added to the tuber or leaf samples at the beginning of an extraction are presented in table 2. Recoveries were always higher with the 5% acetic acid extraction procedure. This was especially evident with the leaf samples for which average recovery ranged from 22.5 to 93.1% for the bisolvent and 5% acetic acid extraction procedures, respectively. Recovery of α-solanine from samples of tuber tissue ranged from 70.4 to 82.4% for the bisolvent and 5% acetic acid extractions, respectively.

When eluate recovered from 'Kennebec' foliage extracted with 5% acetic acid was subjected to TLC, spots equivalent in Rf and color to α-solanine and α-chaconine were recovered. Halos appeared above the α-solanine and α-chaconine spots; these halos corresponded in position

and shape to α and β-solamarine as previously described (1, 2, 6). Authentic tomatine was also added to 'Kennebec' foliage and the resulting mixture was subjected to the 5% acetic acid extraction and TLC procedure. Tomatine was not recovered by this extraction procedure based on the TLC study.

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Table 1. Glycoalkaloid concentration in leaves and tubers of several potato cultivars determined with the bisolvent and 5% acetic acid extraction procedures.

Source of Tissue	Total glycoalkaloid (mg)	
	Bisolvent	5% Acetic Acid
Katahdin tuber[a]	7.82 ± 1.51[b]	8.23 ± 0.59
Norland tuber	4.71 ± 1.62	3.94 ± 0.38
Kennebec tuber	8.67 ± 1.32	9.36 ± 0.61
Norland leaf[c]	0.23 ± 0.17	2.47 ± 0.17
Kennebec leaf	0.18 ± 0.08	5.38 ± 0.36

[a]values for tuber tissue reported as mg TGA/100 g fresh weight

[b]all values are reported as the mean of four determinations ± the standard deviation

[c]values for leaf tissue reported as mg LGA/g dry weight

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Table 2. Percent recovery of authentic solanine added to foliage and tubers of potato tissue as determined by the bisolvent or 5% acetic acid extraction procedure.

Extraction Procedure	Source of Tissue[a]	Tissue Glycoalkaloid (mg)[b]	Added Solanine (mg)	Glycoalkaloid Recovered (mg)	% Recovery[c]
Bisolvent	tuber[d]	0.26	0.50	0.53	69.7
"	tuber	0.26	1.00	0.84	66.7
"	tuber	0.26	1.50	1.31	74.4
"	leaves[e]	0.22	0.50	0.21	26.9
"	leaves	0.22	1.00	0.26	21.3
"	leaves	0.22	1.50	0.33	19.2
5% Acetic Acid	tuber	0.34	0.50	0.69	82.1
"	tuber	0.34	1.00	1.07	79.8
"	tuber	0.34	1.50	1.57	85.3
"	leaves	2.76	0.50	3.15	96.6
"	leaves	2.76	1.00	3.44	91.5
"	leaves	2.76	1.50	3.88	91.1

[a] the tissue extracted from the bisolvent and 5% acetic acid systems were from different sources

[b] values for the amount of tuber and leaf glycoalkaloid in potato tissue were the mean of four determinations

[c] % recovery was calculated from the following equation

$$\% \text{ recovery} = \frac{\text{glycoalkaloid recovered}}{\text{tissue glycoalkaloid + added solanine}}$$

[d] tuber tissue sample size for extraction was 20 g fresh weight

[e] leaf tissue sample size for extraction was 0.25 g dry weight

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Discussion

When analyzing for TGA, the 5% acetic acid extraction system and the bisolvent method are comparable. The former procedure improved slightly both recovery of authentic solanine and the precision of the analysis. The 5% acetic acid extraction only recovers ammonia precipitable glycoalkaloids while the bisolvent extraction system is theoretically much more comprehensive in nature. Since solanine and chaconine are the major glycoalkaloids present in potato tissue and both are ammonia precipitable, either method could be applicable for most TGA analyses.

The bisolvent extraction was unsuitable for quantitative recovery of LGA (Table 2). In contrast, the 5% acetic acid extraction procedure yielded high rates of recovery of authentic α-solanine. Recovery of authentic α-solanine was linear over the range of concentrations considered. When either extraction method was employed, the percent recovery of authentic α-solanine from leaf tissue appeared to decrease with increasing concentrations of the glycoalkaloid; the differences in percent recovery, however, fall within experimental.

With this method the major glycoalkaloids present in potato tissue, via. α-solanine, α-chaconine, and the solamarines are recoverable as determined by TLC. Since authentic solanine is not available commercially, many researchers use the more readily available tomato glycoalkaloid, tomatine, as an internal standard. It is important to note that for undetermined reasons tomatine could not be recovered with the 5% acetic acid extraction procedure.

In light of recent criticisms of the bisolvent extraction concerning low and variable recoveries of authentic solanine (5), the 5% acetic acid procedure may be the extraction of choice when dealing with potato cultivars known to contain mainly ammonia precipitable glycoalkaloids. Hydrolysis, subsequent benzene extraction and determination of the aglycones is effective and highly reproducible. However, this procedure is quite time consuming and the search should continue for faster and more reliable methods for quantifying glycoalkaloids.

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